

Characterization of Type I Collagen Fibrillogenesis Using Atomic Force Microscopy

**An Undergraduate Honors Thesis
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ABSTRACT

Bone is a ‘smart’ material adapting its material composition and properties in response to external loading to maintain the mechanical integrity. Thus, understanding the mechanism to control its adaptive behavior is extremely useful not only for improving clinical treatment but also for mimicking its design strategy for boundless engineering applications. As one of bone’s main constituents, collagen type I is the most abundant protein consisting of osseous tissue. The bio-mineralization process of collagen template controls the mechanical stiffness of bone in response to external loading. While the involvement of collagen in the bone mineralization process is widely accepted, its role and mechanism have not been fully understood yet. As a long-term goal of this research, we aim to investigate the role of collagen in transducing the mechanical input to the biological signal in the process of bone mineralization. For this purpose, it is required to fabricate the collagen template in vitro with varying density and fibril arrangements in a controlled fashion. Here, in vitro fibrillogenesis of the collagen is investigated depending on the fabrication conditions such as ion concentration, pH level, and collagen concentration. Atomic Force Microscopy (AFM) is employed to characterize the growth of fibril width, alignment, and density of the assembled collagen matrix. The self-assembly property of the collagen on various conductive substrates such as gold and platinum are also studied to prepare the collagen-coated samples for future research of piezoelectric property of the collagen.

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Chapter 1: Introduction

1.1 Bone and Bone Mineralization

Bone, one of the most indispensable parts of our body, has important functions and utilities for supporting our body securely and effectively. Bone structures are able to resist external mechanical loads and provide adequate supports to our body. Bone is also a smart material in that its composition and structure are remodeled to adapt to various loading conditions to provide an ideal condition. Thus, studying the composition and material properties of bone is significantly important not only for clinical studies, but also for engineering applications. Bone is a heterogeneous and hierarchical material which is formed by basic structural units that contain bone packets and individual osteons (Eriksen, Axelrod, & Melsen, 1994). The basic structural units are constructed by the modeling and remodeling process involving bone cells activities to create the collagenous tissues that are gradually mineralized (Roschger, Paschalis, Fratzl, & Klaushofer, 2007). As a result, the basic structural units have well-arranged collagen fibrils and deposited minerals (Weiner, Traub, & Wagner, 1999) to organize the bone into tough but also flexible structures.

The formation of the bone structures is importantly contributed by the type I collagen which is known to be the most abundant protein consisting of osseous tissues in all animals. Type I collagen functions as a basic building block that helps achieve the complex formation of bone hierarchy structure as shown in Figure 1. The mechanical strength that the bone structures have exhibited in different length scales is due to the minerals such as hydroxyapatite (HA) entangled with collagen fibrils (Denning, 2014). As it can be seen in Figure 1, the components of a hierarchical bone structure are shown from macro scale to micro scale. In nanoscale, collagen molecules form into collagen fibrils and store the mineral contents in the hole zones. The collagen fibers then form into the network (Haversian canal) which contains in each osteon and

finally build up into bone structures. Recent studies have demonstrated that collagen fibrils' capability of being mineralized is a key property for providing the shape, mechanical support, and strain energy storage in bone (Li, Asadi, Monroe, & Douglas, 2009). It is widely accepted that the network that type I collagen fibrils form serves as a template for the mineralization process. However, its specific role and mechanism has not been well understood yet.

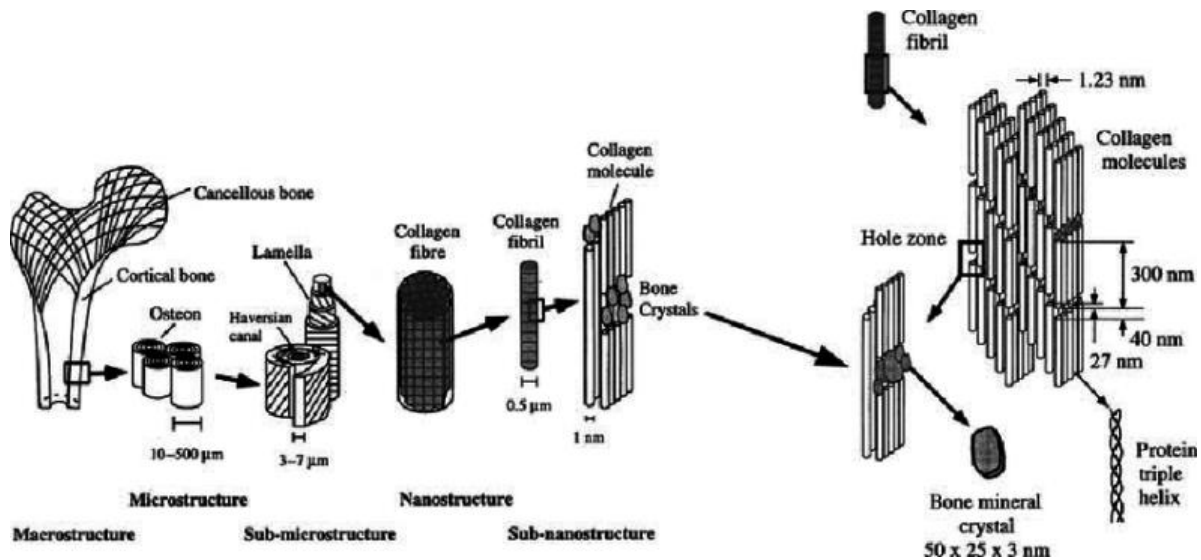


Figure 1: Hierarchical structure and components of bone with length scales. Adapted From [11]

In general, bone structures consist of approximately 25% type I collagen and 75% inorganic mineral depositions (mainly hydroxyapatite). Type I collagen as a soft and organic part provides unique properties and mechanisms for the mineral depositions. The inorganic minerals as a stiff part provides structure supports and stiffness of the bone. Previous studies have indicated that the coordination between type I collagen and inorganic minerals contributes to the bone (re)modeling process. In macro scale, it has been generally accepted that bone is a dynamic organ that can adapt to various mechanical environmental conditions (Martin, Burr, & Sharkey, 1998). This can be concluded by the “Wolff’s Law”, stating that under increasing loading on a particular bone, the bone will make itself stronger by remodeling itself to resist the increasing

mechanical loading. However, if the loading becomes weaker, the bone will also become weaker because of the lack of stimulus for it to remodel (Ahn, & Grodzinsky, 2009). In micro scale, however, the specific mechanism of how bone cells perceive and respond to mechanical loads for the remodeling and mineralization process has not been well understood (Ahn et al., 2009).

Strain-generated potentials (SGP) has been a compelling mechanism for bone remodeling process that widely accepted by the scientific community since it was observed by investigators in early 1960s that there were electrical potentials differences generated along the lateral and longitudinal axes of the bone under mechanical strain (Friedenberg, & Brighton, 1966). There have been two paradigms developed and studied that attribute to SGP and the mineralization process of bone. The first one is based on the collagen piezoelectricity and the second one is based on the streaming potentials. However, previous studies and experimental findings have indicated that streaming potential was a more plausible drive for SGP (Ahn et al., 2009). It is because the streaming potential is demonstrated to generate stronger charges under stress with relaxation times considerably longer than the relaxation times generated by collagen piezoelectricity. Therefore, the piezoelectricity of collagen became a less compelling mechanism for explaining the SGP in bone (Ahn et al., 2009). Even though collagen piezoelectricity has become a less favorably explanatory mechanism in SGP, as the advancement of nanotechnology and a lot more research was geared towards understanding the role of piezoelectricity of collagen for SGP mechanism in bone. We are able to take a new look and conduct more research on the piezoelectricity of collagen fiber in micro scale to explore its specific roles and contributions in the mineralization process of bone structures.

1.2 Objective of the Research

For this research, the techniques of Atomic Force Microscopy (AFM) was applied to study collagen fibrils because AFM is a useful tool for studying biomaterial's properties in nanoscale. Plenty of previous research has studied the morphology, piezoelectricity, mechanical properties, and fibrillogenesis of collagen fibrils. However, most of the properties were only characterized from the static reaction of collagen fibrils. In the future of this research, a dynamic environment will be created to link collagen piezoelectricity to the mineralization process.

In long-term research, type I collagen will be coated on a conductive micro beam structure. Then we can apply force loads and also electric potentials to the micro beam coated with collagen fibrils to study the piezoelectricity. This micro beam structure will also be placed into simulated body fluid (SBF) which contains similar mineral compositions with our blood to create the same physiological conditions in human body for the collagen fibrils while mechanical loads are applied to the collagen to observe and characterize its mineralization mechanism. Many studies have indicated that the orientation of collagen fibrils and fibers and the axis of mineral contents can be dependent on the direction of loading (Skedros et al., 2006; Granke et al., 2013; Georgiadis et al., 2016). Therefore, characterizing the orientation and alignment of in vitro collagen self-assembly will be important to understand the role of collagen piezoelectricity in response of various loading conditions and directions. In this research, the in-vitro self-assembly of collagen fibrils on conductive substrates was characterized with respect to different ionic strength, pH, and substrates in a controlled fashion as an initial step of future research.

1.3 Significance of Research

Collagen as one of the most important components in animal body and especially bone

structures has a huge amount of meaning of studies. Its various properties and mechanisms have important utilization in engineering and clinical fields. Here, the fibrillogenesis of collagen was studied to figure out the patterns that the self-assembly of type I collagen matrices grow on conductive substrates. This research can also identify and classify the effect of pH and the ionic components such as K^+ in the buffer solution on the self-assembly of type I collagen. These factors were studied to predict the effect so that the rule of preparing self-assembly of collagen can be better understood. As mentioned in the objective section, the reason of characterizing collagen self-assembly on conductive substrates is to pave the way for future research on studying the piezoelectricity and mineralization process of the self-assembly of type I collagen.

1.4 Overview of Thesis

This thesis contains 5 chapters. Chapter 2 will introduce a more detailed background of collagen piezoelectricity, collagen self-assembly and Atomic Force Microscopy (AFM). The experimental methods applied for preparing collagen self-assembly on conductive substrates and AFM imaging methods will be introduced in Chapter 3. Chapter 4 will list the results obtained through multiple experiments and Chapter 5 will conclude and summarize the key contributions of this thesis and discusses the future applications and directions of this study.

Chapter 2: Background and Literature Review

2.1 Type I Collagen and Its Piezoelectricity

The most common structure in which collagen and collagenous tissues form is a rope like fiber with a diameter of a few hundreds of nm and a length of up to hundreds of microns.

Collagen fibrils can form into different sizes, different orientations and structures with the integration of inorganic components to finally build into various tissues such as tendon, bone, cartilage, skin, blood vessels, and etc. (Denning, 2014)

Each collagen molecule has polar bonds, characterized by an amine (NH_2 or N terminal) and carboxyl ($COOH$ or C terminal) terminus at either end. This results in a dipole moment (or polarization) along the long axis of the molecule which is directed from the N to C termini as shown in Figure 2 (Kadler, Holmes, Trotter, & Chapman, 1996). Each collagen molecule assembles laterally through electrostatic interactions and hydrophobic interactions and thus most of the collagen molecules will form into fibrillary structures (Wallace, 1990). This makes each molecule placed from one another by around 67 nm longitudinally. The fibril formed in the end has a repeated gap and overlap structure of the D-periodic distance which is 67 nm (Denning, 2014). The topographical look of a type I collagen fibril with its D-periodic spacing captured using Atomic Force Microscopy is shown in Figure 3.

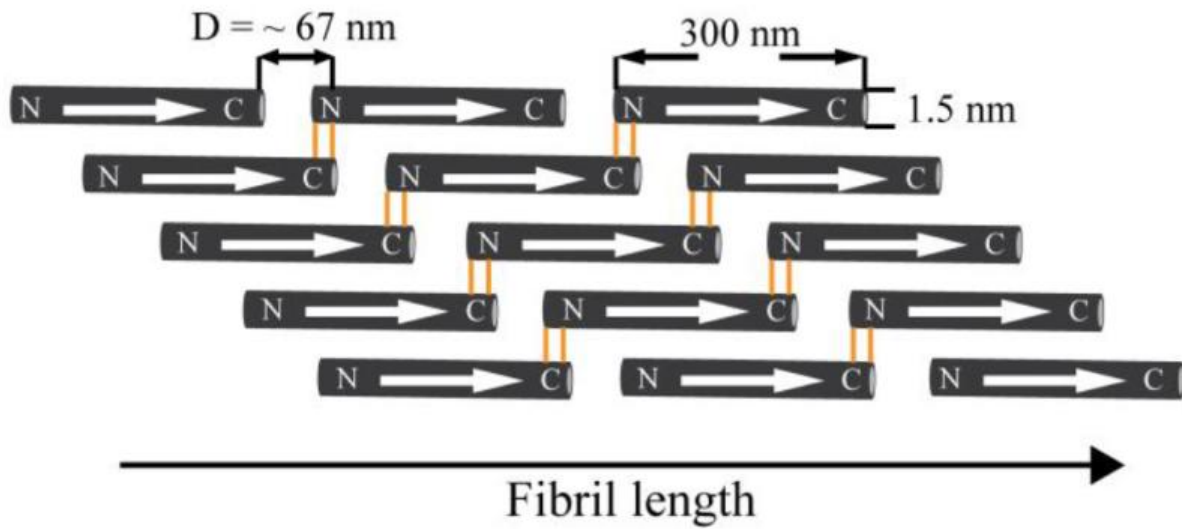


Figure 2: Molecular structure of a type I collagen fibril. Adapted from [3]

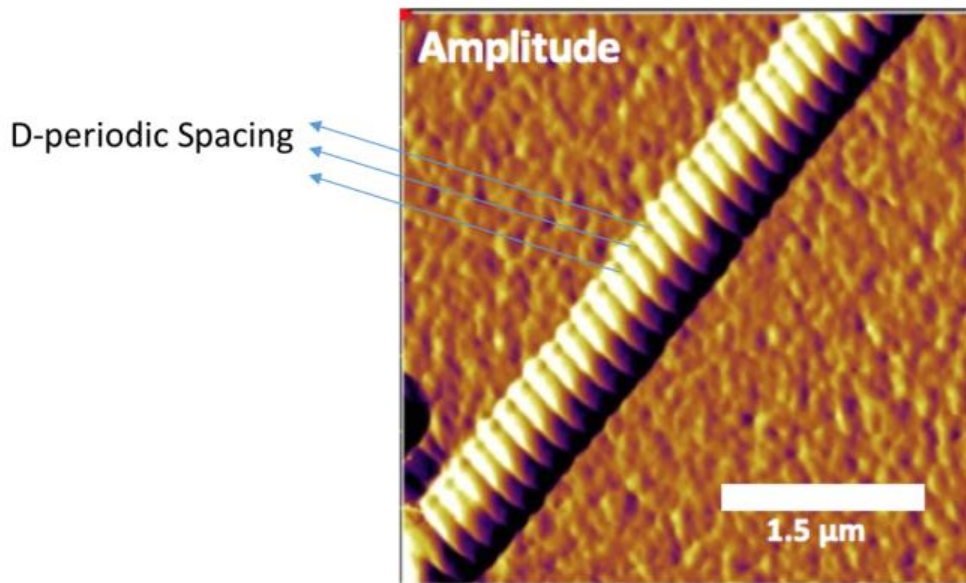


Figure 3: The topographical image of a single type I collagen fibril captured using AFM.

Type I collagen has important functional properties such as piezoelectricity. Piezoelectricity is a property of non-centrosymmetric materials that generating charges when a

mechanical load is applied. Conversely, they deform and generate mechanical strains when an electric field is applied. As introduced in the Chapter 1, piezoelectricity is one of the potential mechanisms for SGP participating in the bone mineralization process. Collagen piezoelectricity is also an important property for the purpose of future study of this research. The piezoelectric effect of collagen fibrils can be characterized at the same scale as the process occurs in physiological conditions. Many previous studies have characterized the piezoelectricity of collagen fibrils by applying the techniques of Piezoresponse Force Microscopy (PFM). PFM can collect and characterize the piezoelectric responses of individual collagen fibrils in both their radial and axial directions through operating in lateral and vertical response modes (Minary-Jolandan ,& Yu, 2009). As shown in Figure 4, while the AC voltage is applying to the conductive substrate coated with collagen fibrils, due to the piezoelectric effect, strain generated in lateral and vertical directions by the collagen fibrils then will be measured by the deflection of the AFM probe. Through multiple previous experiments, collagen has been proved to have anisotropic piezoelectric effect and comparatively dominant in shear direction.

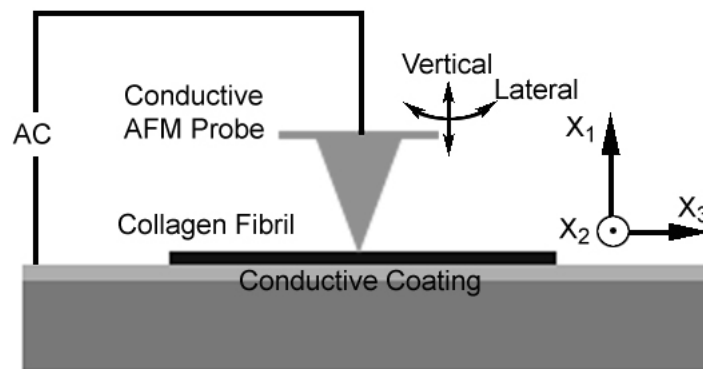


Figure 4: Schematics of the basic set-up of PFM. Adapted from [9]

2.2 Collagen Self-Assembly

Previous studies have confirmed that collagen molecules are able to self-assemble into collagen fibrils that can be obtained and prepared on substrates with appropriate environmental conditions (Zhu, & Kaufman, 2014). Properties such as morphology, mechanical stiffness, and fibrillogenesis of the collagen self-assembly can also be varied with the application of different conditions such as ionic strengths, pH levels, and temperatures. Having collagen self-assembly on a substrate allows us to conduct various in vitro studies to explore its properties and applications (Jiang et al., 2004). As nanotechnology becoming more advanced, we are able to characterize collagen self-assembly precisely by using AFM.

2.2.1 Effect of K^+ Concentration and pH on Collagen and Substrate Interactions

Previous studies have frequently shown the ionic strength of K^+ and pH-level have a significant effect on the structural formation of collagen self-assembly on mica substrate. They have also indicated that the ionic strength of K^+ and pH can generate a coupling effect on interactions between collagen fibrils themselves and interactions between collagen fibrils and the mica substrates. The interactions are related to the affinity between collagen and substrate which can affect the orientation and arrangement of collagen self-assembly. Through the comparison of the morphologies of collagen fibrils model between in molecular dynamics (MD) simulations and in AFM experiments (Figure 5) in a previous study, the collagen assembly on mica substrate was confirmed to be governed by the competition between the collagen-collagen (c-c) interactions and the collagen-substrates (c-s) interactions (Narayanan et al., 2014). The study has indicated that in general, if a sample has strong c-s interactions but weak c-c interactions, the collagen fibrils will have a strong affinity toward the surface but weak affinity between themselves thus create random monolayer alignments. However, if a sample has strong c-c

interactions but weak c-s interactions, the collagen fibrils will have a strong affinity between themselves but a weak affinity toward the substrate which will cause the collagen fibrils form into 3-D bundles (Narayanan et al., 2014). The interactions have been proved to be affected by different pH and ionic strength conditions and patterns have shown that stronger ionic strength and higher pH will increase the c-c interactions and decrease the c-s interactions (Narayanan et al., 2014). Therefore, by varying the ionic strength of K^+ and pH of the buffer solution, we are able to control the c-c interactions and c-s interactions to obtain different morphologies of collagen self-assembly.

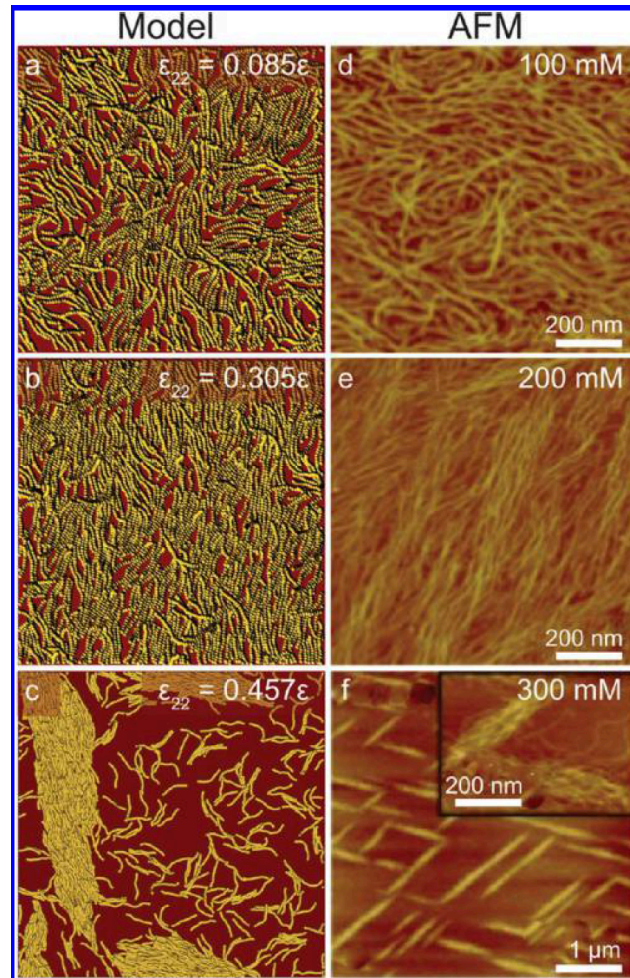


Figure 5: Comparison of the collagen self-assembly morphology on mica between MD simulations (a-c) and AFM images (d-f). KCl concentration (ionic strength) with pH 4.0 condition increases from 100 to 300 mM from image d to image f. Adapted from [14]

2.2.2 Roles of Collagen Self-Assembly in This Research

In this research, collagen self-assembly on conductive substrates were investigated. Most of previous studies have well studied the collagen self-assembly on mica or non-conductive substrates. However, the formation of the self-assembly of collagen on conductive substrates remains to be unanswered. As introduced in Chapter 1, the future application will utilize the in vitro fabrication of collagen fibrillogenesis through collagen self-assembly on conductive substrates such as gold and platinum to study the piezoelectricity and mineralization mechanism of type I collagen. By varying the ionic strength and pH conditions, the morphology of collagen self-assembly on conductive substrates can be controlled and characterized.

2.3 Atomic Force Microscopy

AFM is one type of scanning probe microscopes (SPM). It has been an important nanoscale characterization tool since it was invented by Gerd Binnig, Calvin F. Quate and Christoph Gerber in 1986 (Wallace, 1990). AFM utilizes the force interaction between a sharp probe tip attached to the end of a cantilever beam and the sample surface to create a 3-dimensional topographical image. The advancement of AFM allows us to have nanoscale characterization of insulators and biomaterials (Denning, 2014).

The state-of-the-art AFM (Asylum Research MFP-3D-Infinity) was used for this research in Micro/Nano Multi-physical Dynamics Laboratory (Figure 6&7).



Figure 6: AFM scanner and sample stage.

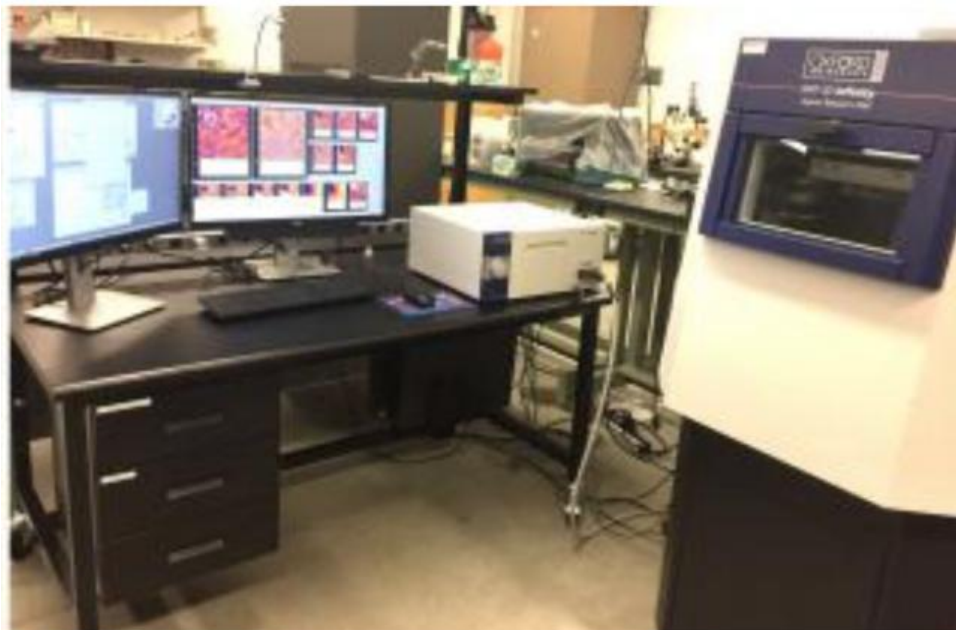


Figure 7: AFM system. Adapted From [12]

The resolution of AFM is based on the sharpness of the probe and it is dependent on the other experimental settings such as imaging environment, imaging mode, sample flatness, noise level, and etc. The typical lateral resolution of AFM is around 1-10 nm and vertical resolution of around 0.1 nm. Its operation is based on the mechanism illustrated in Figure 8. The main components are the sensor (cantilever), detector (laser and photodiode) and controller (feedback loop). As the cantilever interacts with the sample surface and scans over the desired region, the laser beam is reflected from the cantilever tip to the position-sensitive photodiode detector. Then the surface of the sample can be tracked for imaging based on the position of the laser beam used in the feedback loop.

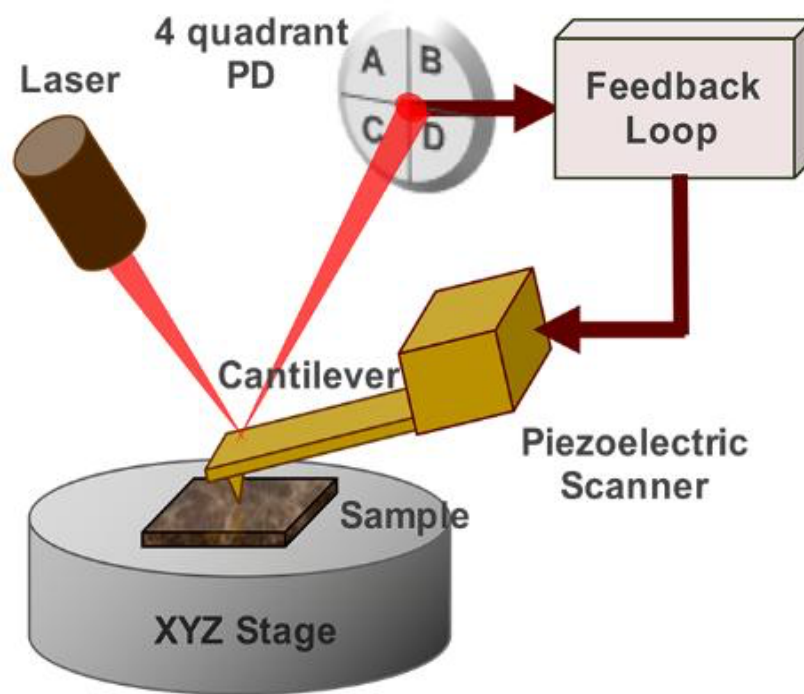


Figure 8: AFM operational components schematic. Adapted from [12]

A resultant cantilever deflection is caused by the force interaction between the AFM tip surface as they get into contact. The relationship of the resultant deflection and tip to surface interaction can be concluded by the Hooke's law:

$$F = -kx \quad (1)$$

where F is the force interaction between the tip and the surface, k is the spring constant of the cantilever beam and x is the deflection of the cantilever beam from its original position. As can be seen from equation (1), the spring constant k of the cantilever beam can be utilized to change and control the force exerted on the sample surface, which allows us to change cantilever beams with different spring constant according to the softness of different biological samples (Denning, 2014).

AFM has various scanning modes to obtain topographical images in various medium. Most commonly used modes are contact mode and tapping mode, which can scan the material sample either in air or in liquid. For this research, the tapping mode AFM was used and applied for collecting the topographical images of the collagen self-assembly samples in air. The details of tapping mode will be introduced in the next section.

2.3.1 AC Tapping Mode in Atomic Force Microscopy

The tapping mode AFM has a dynamic imaging mechanism while scanning the sample. In tapping mode AFM, the cantilever oscillates at a certain frequency and amplitude and the tip has contact with the surface only for a short time for each time it touches the surface. Therefore, the issue of lateral forces and drag across the surface then can be solved and images with higher resolution can be collected. The operation of the tapping mode AFM is shown in Figure 9. The figure shows the shift of the resonance curve of the cantilever as the cantilever gets close and has contact with the surface.

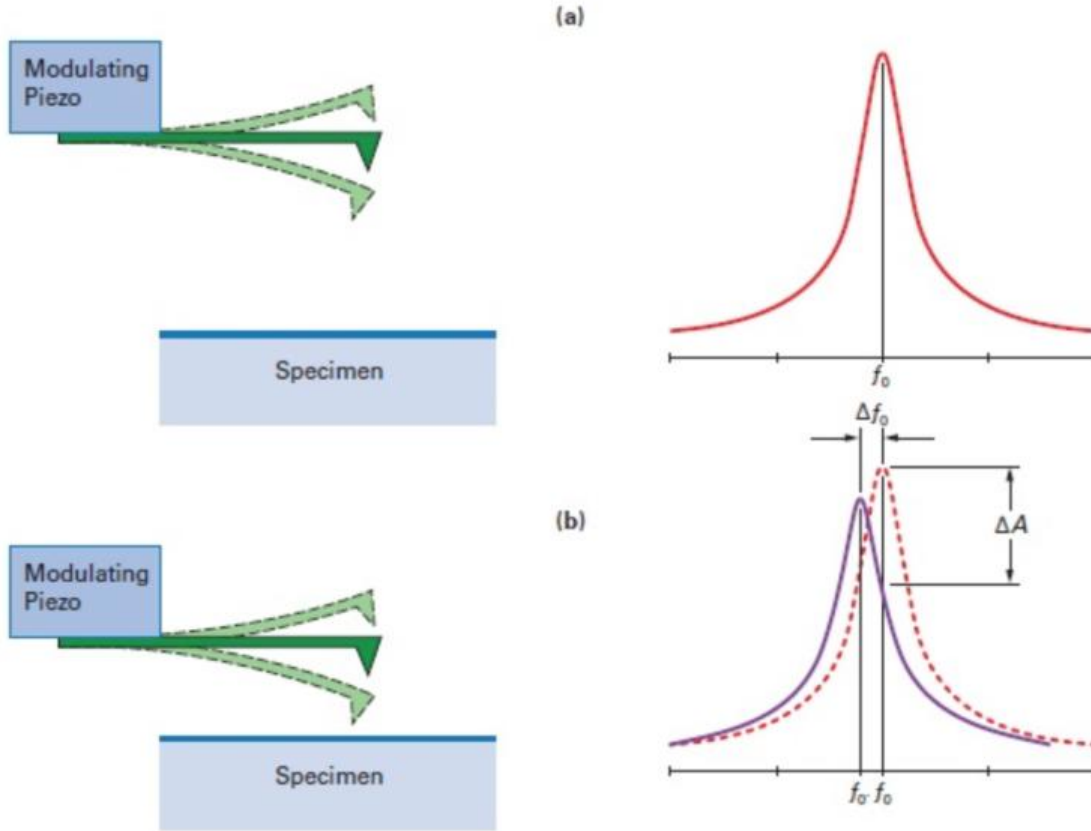


Figure 9: The resonance frequency of the cantilever before touching the surface (a). The shift of resonance frequency of the cantilever after touching the surface (b). Adapted from [18]

The interaction forces between the cantilever tip and the surface dampen the cantilever energy when the cantilever tip starts approaching the surface. Therefore, the amplitude of the cantilever will decrease, and the change of the amplitude will be used as feedback signal in the feedback loop for imaging. During the scan of the tip over the sample surface with feedback mechanism, PZT scanners enable to maintain the tip at a constant force or a constant height. Thus, as the tip is oscillating up and down with the contour of the surface, the laser beam is deflected from the cantilever which allows the photo detectors to measure the difference of light intensities between its upper and lower part (Mourougou-Candoni, 2012). Additionally, phase images can also be generated in tapping mode AFM. The phase image is measured by the phase

shift between the driving oscillation and the resultant oscillation of the cantilever while touching the sample surface. Because the phase shift is related to the energy loss of the cantilever, the phase images will provide more information about the elastic properties of the sample material. The tapping mode AFM can provide accurate images and information of the sample with minimizing the damage that the cantilever tip could possibly make to the sample. Therefore, this mode was applied to characterize the collagen self-assembly on substrates.

Chapter 3: Experimental Methods

3.1 Design of Experimental Procedure

The rough mechanism of the design for performing the experiment is addressed in this section. The collagen self-assembly and its mineralization on a mica substrate was first performed in video-rate AFM to capture the real-time formation process. This research was then divided into two parts. The first part was to prepare type I collagen self-assembly on three different substrates. Here, the ionic component used in the buffer solution was K^+ in KCl. Then multiple collagen solutions were prepared with buffer solutions with various pH values between 5.0 ~ 9.0 and K^+ concentrations with 200 mM and 300 mM. The collagen was then coated on silicon, platinum and gold substrates by following the procedures discussed in Chapter 3.2.2. The second part was to apply the AFM tapping mode to scan the prepared collagen samples and collect their final morphological images. Finally, by comparing the morphologies of the collagen self-assembly samples on different substrates, the effect of ionic strength, pH, and substrates on collagen fibrillogenesis is characterized.

3.2 Sample Preparation

3.2.1 Preparation of Collagen Solutions

The collagen used for this experiment was rat tail type I collagen solution purchased from Advance Biomatrix Cooperation. This is an as-obtained solution and contains 4 mg/mL of purified type I collagen in 0.2 N Acetic Acid. A phosphate-buffered saline (10 mM) was prepared first and then KCl was added into the buffer to obtain the desired concentrations of 200 mM and 300 mM. The desired pH values (i.e., 5.0, 7.3 and 9.0) were obtained by adding HCl and NaOH into the buffer solution and measured by a pH meter.

10 μ g/mL of collagen concentration was obtained by adding the collagen stock solution (4 mg/mL) to buffer solutions containing 200 mM and 300 mM KCl and 10 mM Na_2HPO_4 in

appropriate volume ratios (collagen/buffer). The collagen solutions were stored in centrifuge tubes and ready to be applied to the substrates for coating.

3.2.2 Coating Collagen Fibrils on Substrates

Rectangular substrates (length ~ 5 mm and width ~ 3 mm) with different materials (silicon, gold, and platinum) were prepared for collagen coating. 6 substrates were prepared for each material. The specific collagen coating setups are shown in Figure 10.

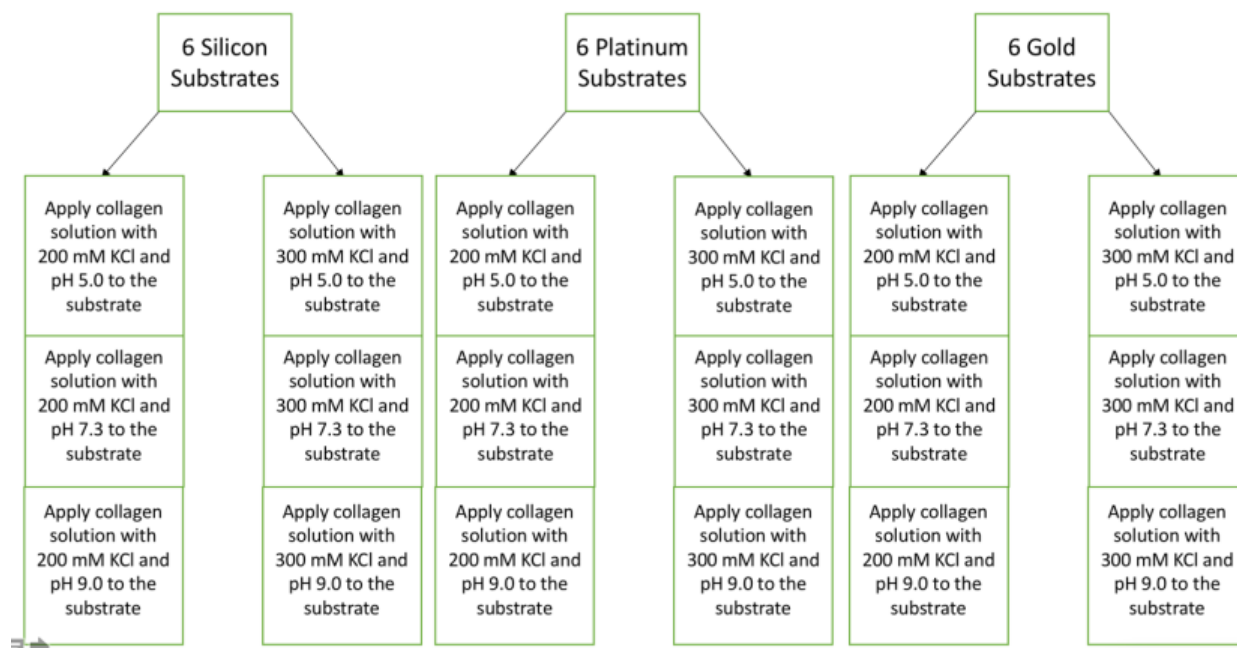


Figure 10: Collagen coating on silicon substrates, platinum substrates, and gold substrates.

The specific coating process was accomplished by the following procedure:

Substrates Cleaning

1. Submerge the substrates in Acetone in a baker and heat up below 55 Degree Celsius for around 10 minutes.
2. Take the substrates out of Acetone and submerge them in Methanol in a baker for around 5 minutes.

3. Take the substrates out of Methanol and rinse them using deionized water for 20 seconds (or place them in a sonicate).
4. Place the substrates on clean glass slides and dry at room temperature.

Collagen Coating Process

1. Gently submerge the substrates in collagen solutions (at room temperature) and leave in contact for 60 minutes.

After Coating

1. Take out the substrates and use deionized water to clean the surfaces for around 15 seconds to rinse off the unnecessary crystals and loosely attached collagen fibrils on the surface.
2. Place the substrates on clean glass slides and dry at room temperature.
3. Carefully glue the substrates onto the prepared glass slides (avoid letting any glue cover the silicon surface).
4. Let the glue dry at room temperature.

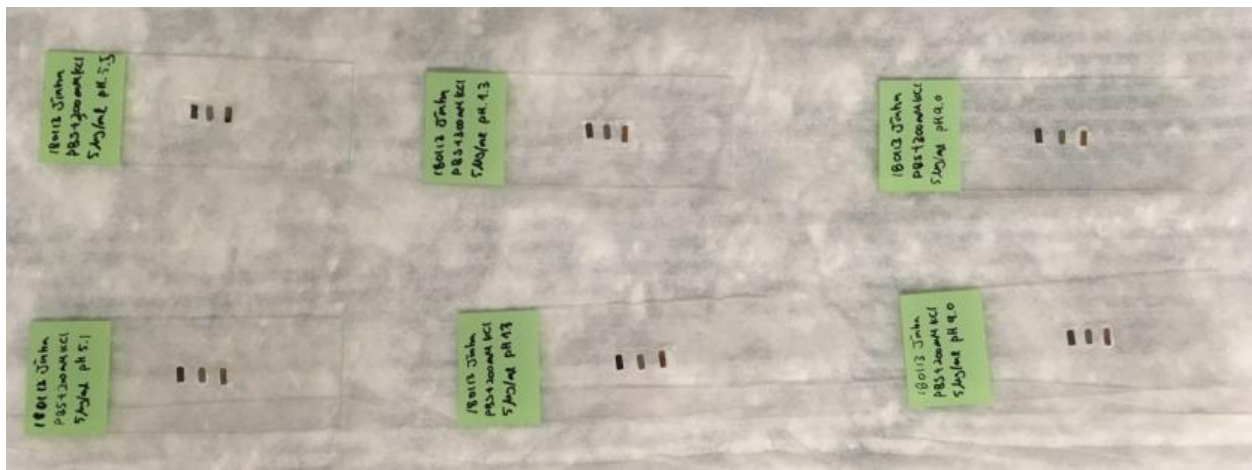


Figure 11: Substrates coated with type I collagen.

The prepared substrates that were ready for AFM imaging are shown in Figure 10. Since the size of the substrates were small, gluing them onto the glass slides allowed the samples to be placed on the stage in AFM. In addition, the substrates were fixed on the glass slides to avoid movement of the substrates while scanning and this was important for collecting good quality AFM images.

3.3 AFM imaging

The in air AFM images were collected in tapping mode at room temperature (23 °C) with the Asylum Research MFP-3D-Infinity AFM using silicon tips AC-240TS-R3 (Oxford Instruments, nominal spring constant 2 N/m and nominal resonance frequency 70 kHz). The drive frequency was 85 kHz, and the drive amplitude was 1.5 V. The scanning rate was 1.5 Hz and the set point amplitude were tuned to around 0.8 V to minimize the force loads onto the collagen surface.

Chapter 4: Results and Discussion

4.1 Overview

The topographical images of the type I collagen samples were collected by AFM. Collagen self-assembly and collagen mineralization images on mica substrates by video-rate AFM was also collected. The results for the first set of samples were collected first. The results have shown that the alignment of collagen self-assembly on platinum substrates more organized than the other substrates in general. Therefore, a more specific experiment (second set) results were collected from the second set of samples which were only platinum substrates. The effect of K^+ concentrations and pH of the collagen buffer solutions on collagen self-assembly will be shown and discussed in the following section. At the same time, the difference of collagen alignments on different substrates will also be shown and compared.

4.2 Collagen Self-Assembly Images Collected by Video-Rate AFM

In Figure 12, the formation of type I collagen self-assembly on a fresh cleaved mica substrate is shown. The collagen fibrils were prepared with a buffer solution with a certain level of ionic strength at neutral condition. The images were collected by the video-rate AFM from Asylum Research which can give us a real-time look at collagen fibrils in nanoscale. As seen in Figure 12, once the collagen solution was applied to the mica substrate, collagen started attaching to the mica surface and forming assembled fibrils. At around 11 minutes from the beginning, self-assembled collagen fibrils almost fully covered the mica surface with an oriented alignment.

These results have shown the collagen self-assembly process on mica substrate as discussed in earlier chapters.

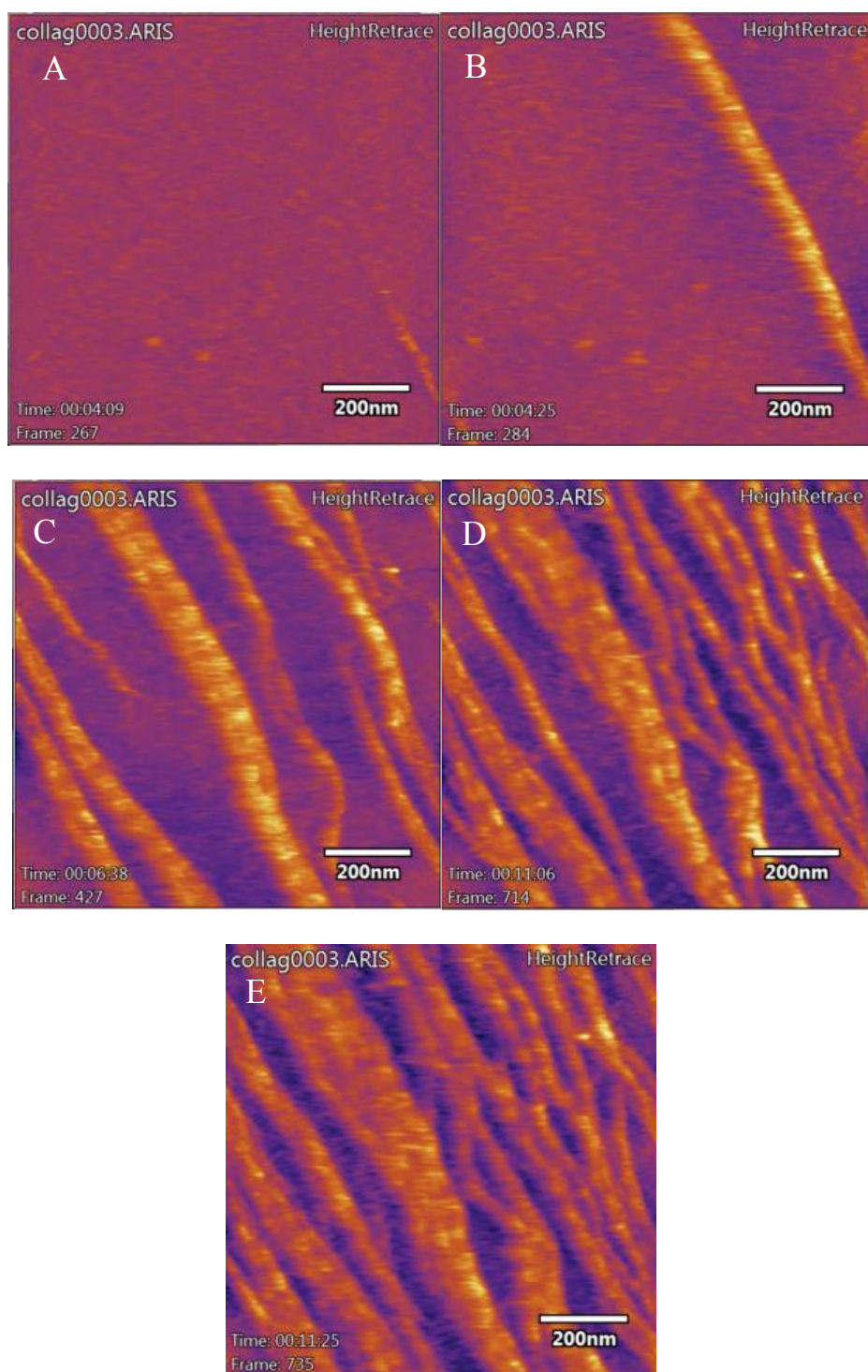


Figure 12: Formation of type I collagen self-assembly by video-rate AFM. (A-E) Topographical images growing collagen fibrils on a mica substrate.

4.3 Collagen Self-Assembly on Conductive Substrates

The AFM images of self-assembled collagen on silicon substrates under conditions of 200 mM and 300 mM of K^+ ions and pH of 5.0, 7.3 and 9.0 are shown in Figure 13 below.

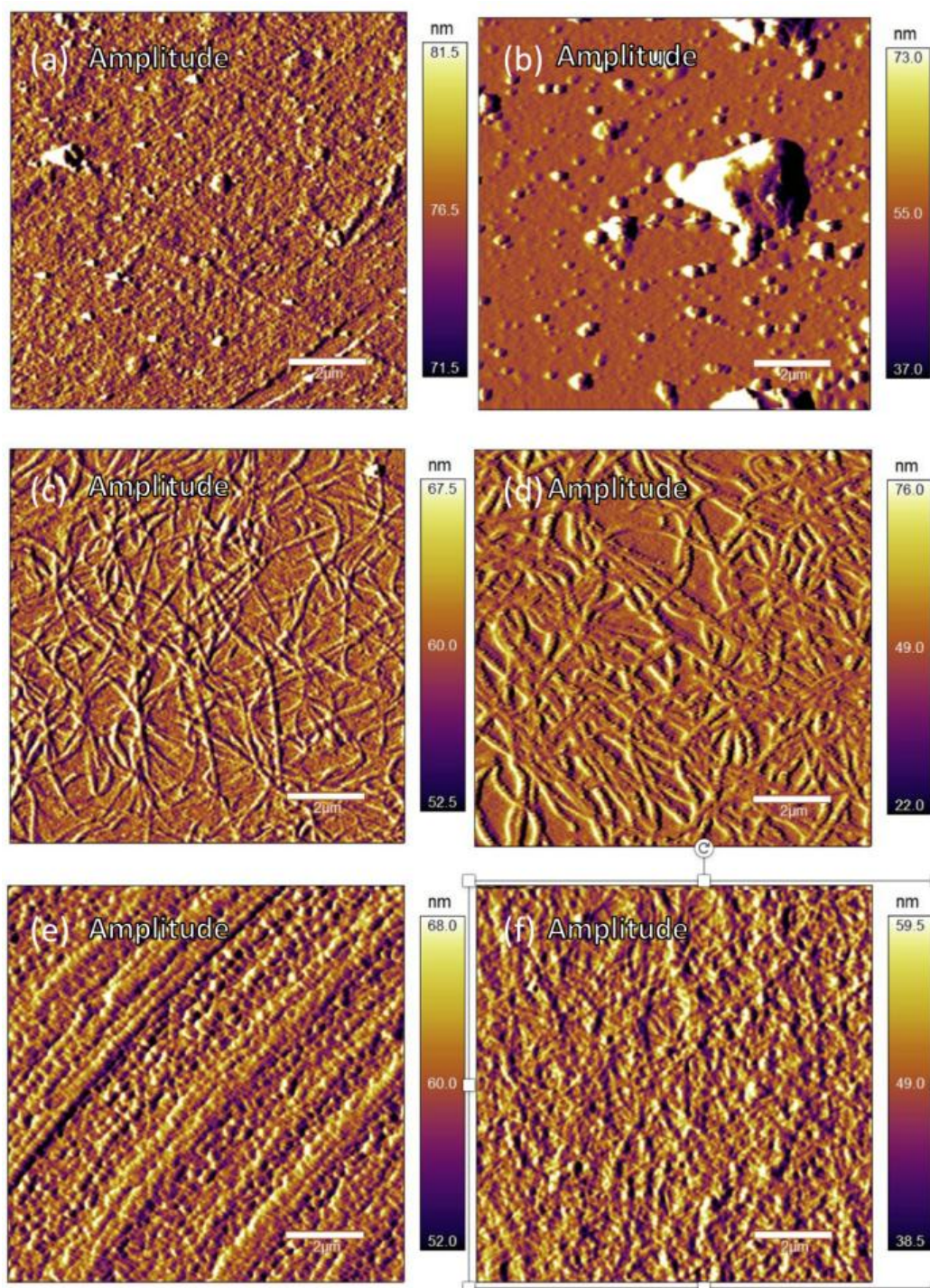


Figure 13: Morphology of self-assembled collagen on silicon substrates collected by AFM tapping mode (a-f). The scale was 2 μm . The pH condition for (a) and (b) was 5.0, (c) and (d) was 7.3, and (e) and (f) was 9.0. The ionic strength was 200 mM KCl for (a)(c)(e) and 300 mM KCl for (b)(d)(f).

As the results seen in Figure 13, in acidic condition (pH = 5.0) there were no collagen fibrils formed on the silicon substrates for both 200 mM and 300 mM KCl. However, as the pH value increased from acidic to neutral and basic, significant amount of collagen fibrils were formed on the silicon substrates. The orientation of the collagen fibrils started to be more uniform as the pH value increased as shown in images from (a) to (c) to (e). Under the same pH condition, as the ionic strength increased from 200 mM to 300 mM KCl the alignment of collagen fibrils changed from 2D arrays to thicker 3D bundles. This change was remarkable under the basic condition (pH = 9.0). These results have shown that the pH condition can affect the orientations of the collagen fibrils and the ionic strength can affect the interactions between collagen and substrate. The assembly was dependent on the balance between the c-s interactions and the c-c interactions. The increase of the ionic strength weakened the c-s interactions and increased the c-c interactions, thus the affinity between each collagen fibrils became stronger and the affinity between the collagen and the substrate became weaker which caused the collagen form into thicker bundles.

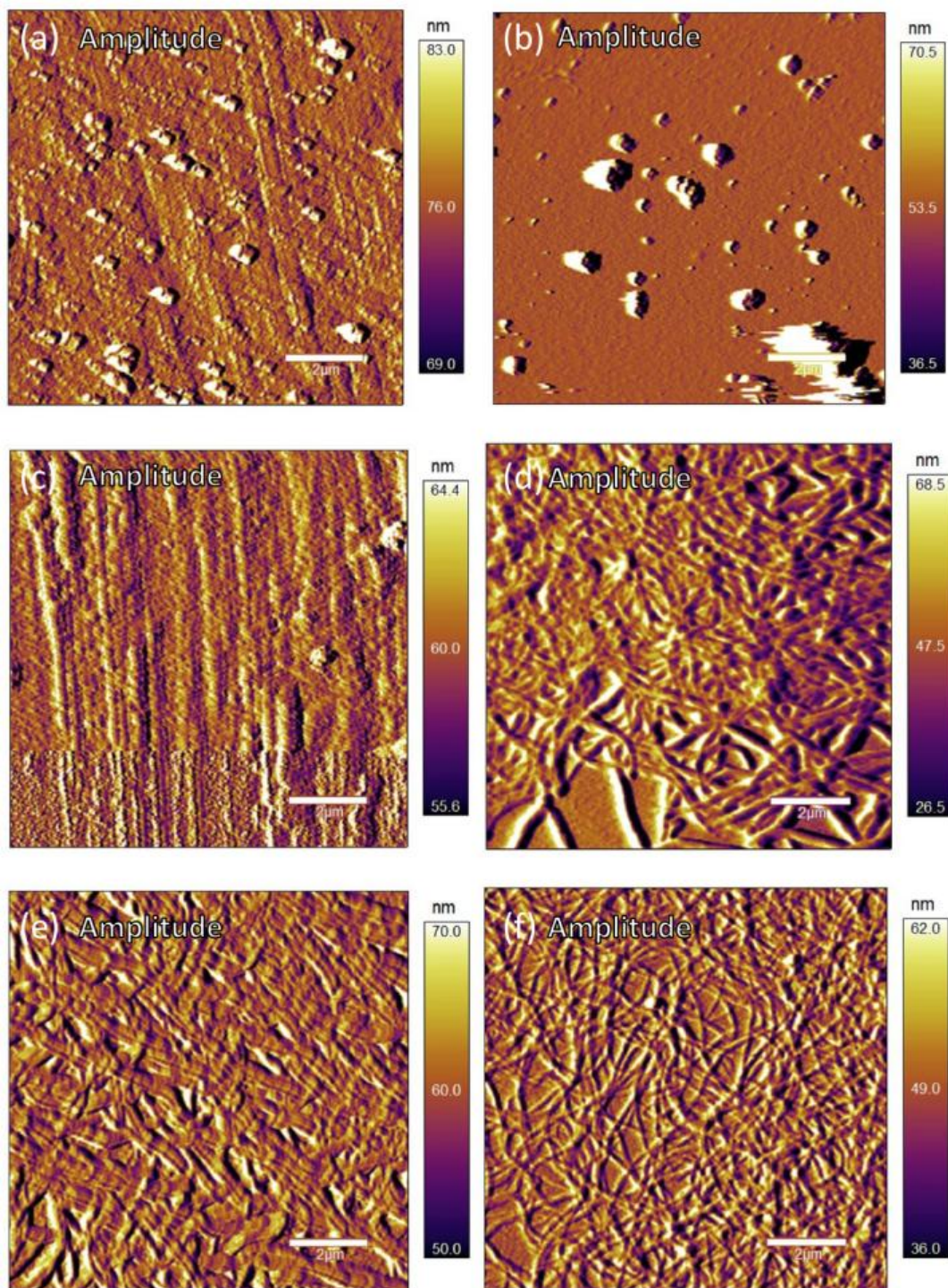


Figure 14: Morphology of self-assembled collagen on platinum substrates collected by AFM tapping mode (a-f). The scale was 2 μm . The pH condition for (a) and (b) was 5.0, (c) and (d) was 7.3, and (e) and (f) was 9.0. The ionic strength was 200 mM KCl for (a)(c)(e) and 300 mM KCl for (b)(d)(f).

Similar results have also shown on platinum substrates shown in Figure 14. The orientations of collagen fibrils became more notable and ordered as the pH value went up especially when the pH is equal to 9.0. Increasing the ionic strength under the same pH value caused the collagen fibrils to form into thicker bundles. The obvious change can be seen from image (e) to image (f) in Figure 12. Image (e) was in 200 mM KCl and pH 9.0. The co-aligned collagen fibrils fully covered the platinum substrate. However, in image (f) which was in 300 mM KCl and pH 9.0, the collagen fibrils formed into 3D bundles.

The morphology of collagen assembly on gold substrates has not shown as consistent patterns as the silicon and platinum substrates. As seen in Figure 15, the only condition that had good collagen alignment was 200 mM KCl and pH 9.0. Other than this condition, no specific or notable patterns were shown of the collagen assembly alignment.

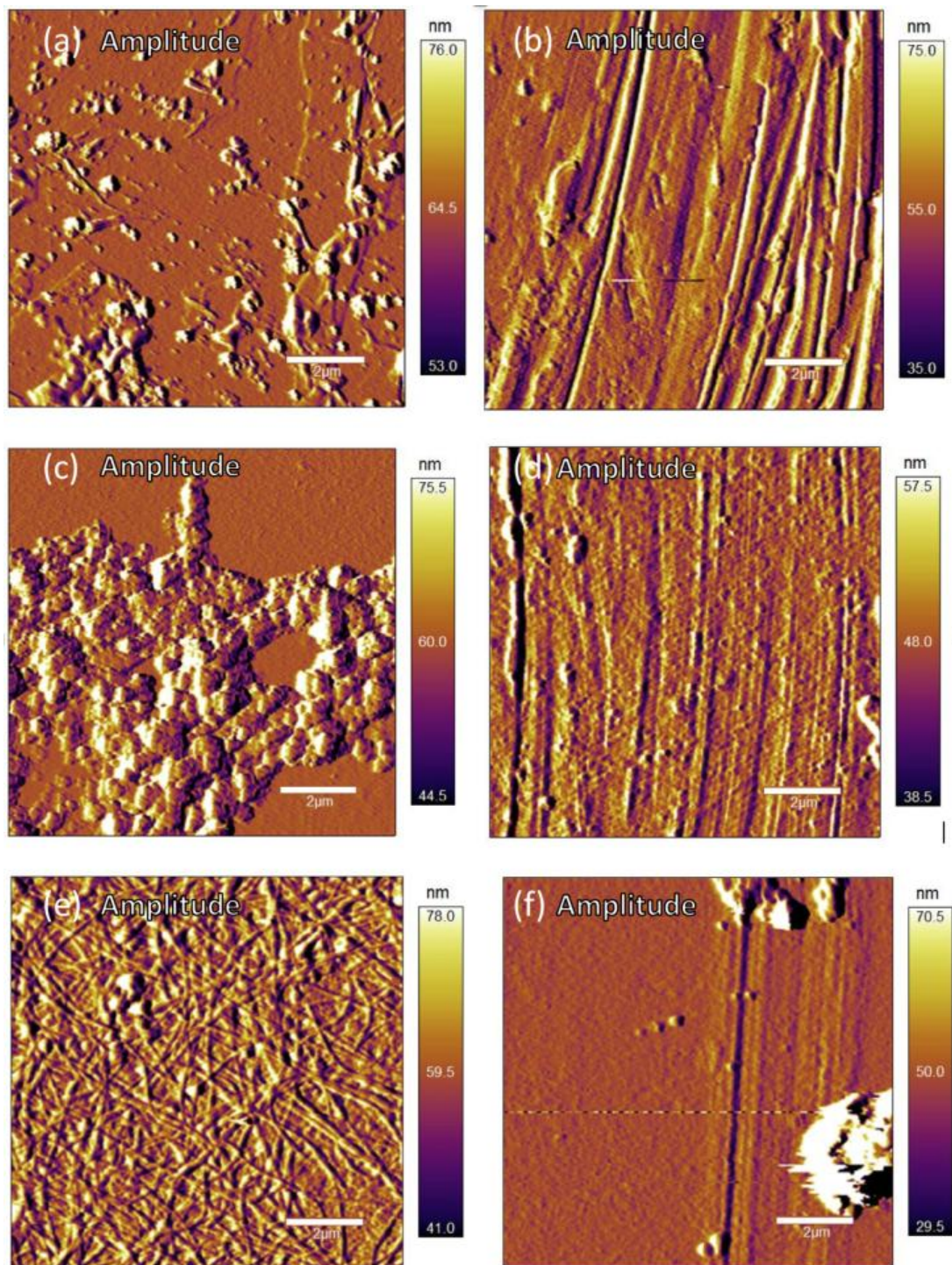


Figure 15: Morphology of self-assembled collagen on gold substrates collected by AFM tapping mode (a-f). The scale was 2 μm . The pH condition for (a) and (b) was 5.0, (c) and (d) was 7.3, and (e) and (f) was 9.0. The ionic strength was 200 mM KCl for (a)(c)(e) and 300 mM KCl for (b)(d)(f).

For all of the three kinds of substrates (silicon, platinum, gold), collagen prepared in condition of 200 mM KCl and pH 9.0 showed comparatively better surface coverage, fibril orientation, and alignments. The change from acidic condition to basic condition affected the orientation of the collagen fibrils. Under the same pH condition, increase KCl concentration from 200 mM to 300 mM led to the collagen fibrils change from 2D arrays to 3D bundles on silicon and platinum substrates.

As the results shown of the collagen self-assembly on silicon, platinum, and gold substrates, platinum and silicon substrates have revealed similar collagen assembling patterns with the changes of pH and ionic strength. However, the results on gold substrates were not very identical. Therefore, the gold substrates were first excluded from the selection for our future experiment. Then as considering the conductivity between silicon substrates and platinum substrates, platinum has better conductivity than silicon and silicon as a semiconductor, its conductivity is dependent on the doping number. Therefore, the best option for continuing our future study of collagen piezoelectricity was platinum substrates.

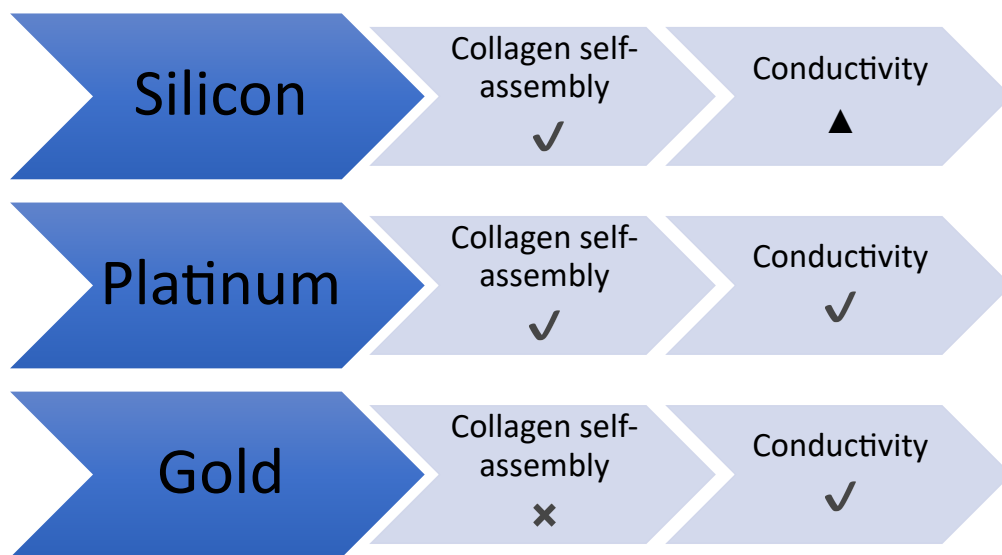


Figure 16: Substrate selection based on collagen self-assembly and conductivity.

The results have also shown that collagen self-assembly formation was more oriented and identical in basic condition (pH = 9.0). The following AFM images shows more specific the effect of ionic strength on collagen self-assembly on platinum substrates in basic condition.

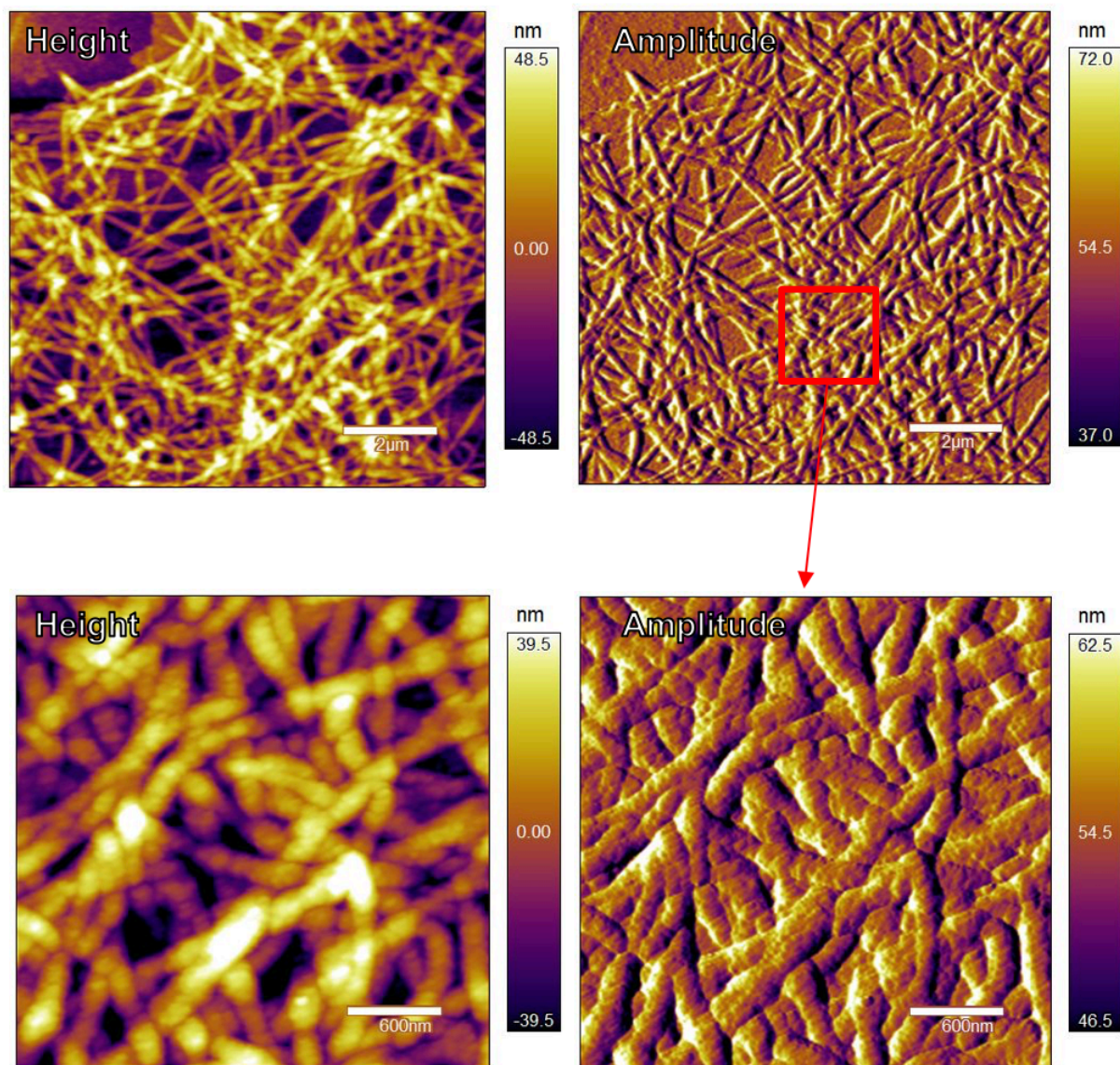


Figure 17: AFM height and amplitude images of collagen self-assembly on platinum substrate with 200 mM KCl and pH 9.0. Images on the top is at 2 μm scale. Images on the bottom is the zoomed in area indicated by the arrow.

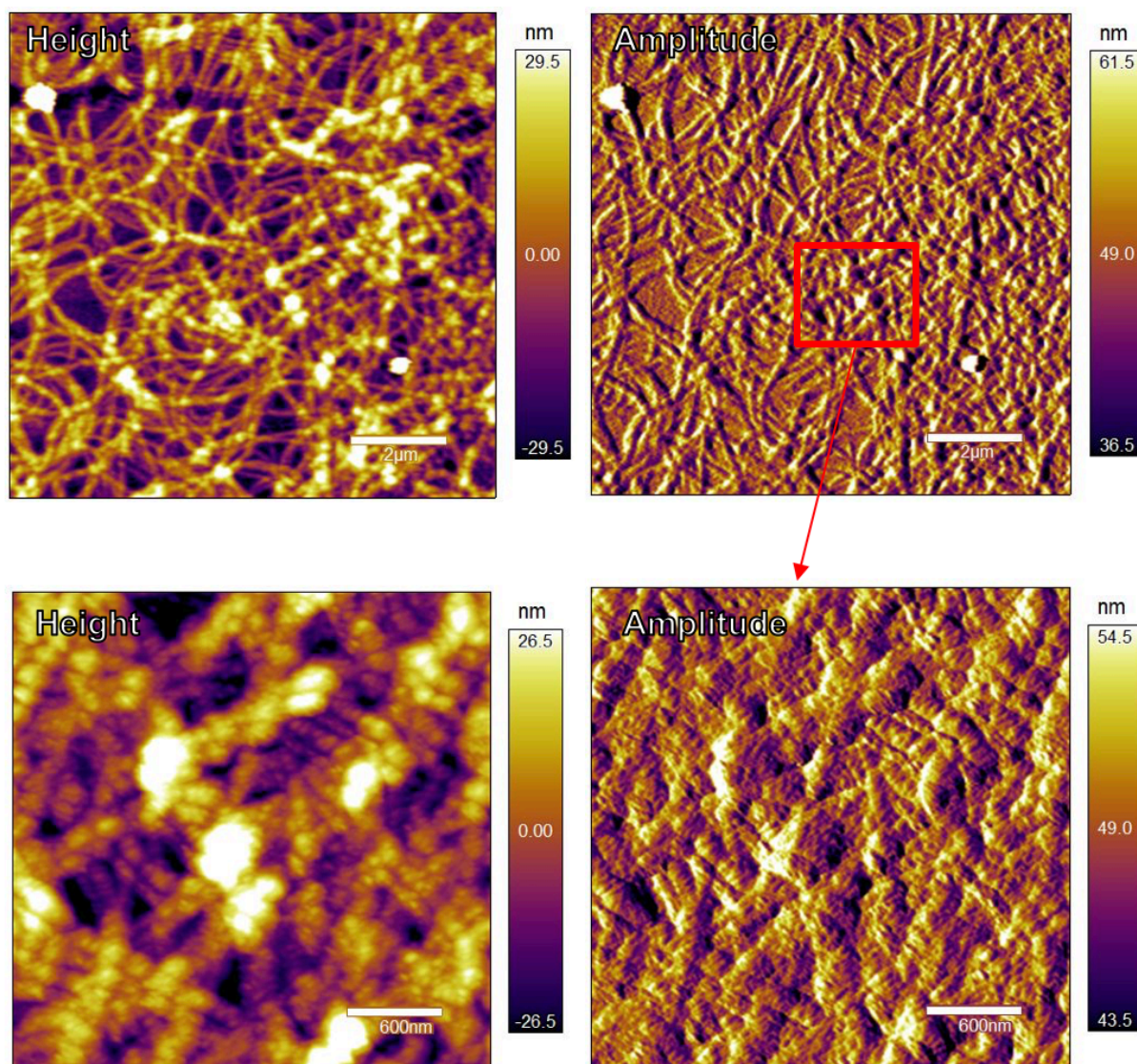


Figure 18: AFM height and amplitude images of collagen self-assembly on platinum substrate with 300 mM KCl and pH 9.0. Images on the top is at 2 μ m scale. Images on the bottom is the zoomed in area indicated by the arrow.

As the zoomed in AFM images of collagen self-assembly shown in Figure 17 and 18, the collagen fibrils with 200 mM KCl were identical and fully formed collagen fibril can be seen covered on the platinum surface. However, as the concentration of KCl increased from 200 mM to 300 mM, the collagen started to bundle up into 3D matrix and its fibrillogenesis was not as identical as with 200 mM. In conclusion, from all of the results, the collagen self-assembly on

silicon, platinum, and gold substrates are characterized with varying the ionic strength and pH levels. The patterns of ionic strength and pH effect on collagen fibrillogenesis were also characterized and platinum substrate was identified as a better option for collagen coating in our future experiment. However, the specific ionic strength and pH conditions for giving the best collagen self-assembly on platinum substrates should be further investigated by varying more parameters and testing more samples.

Chapter 5: Conclusion

5.1 Contributions

Type I collagen as most abundant protein in animal bone structures and tissues plays an important role in supporting our body. Its functionalities and properties are worth to study and there has been a large amount of studies conducted. As the application of nanotechnology became more advanced, we are allowed to characterize collagen fibrillogenesis in nano and micro level. The characterization of collagen fibrillogenesis that has accomplished in this research project helps to understand the patterns of collagen alignments while changing various conditions. A lot of research of studying the properties of collagen are in need of having multiple forms of collagen self-assembly on substrates. Therefore, this research project can provide an insight on the collagen fibrillogenesis with different conditions and preparation of collagen self-assembly on substrates for future research works.

5.2 Future Work

As mentioned in earlier chapters, the use of conductive substrates to conduct the experiments were because of the future work application. The main goal of future work is to study the role of collagen piezoelectricity in its mineralization process. By coating collagen fibrils on platinum micro beam with desired surface alignment, the piezoelectricity of collagen fibrils on the micro beam can be characterized through the experiment setup shown in Figure 19.

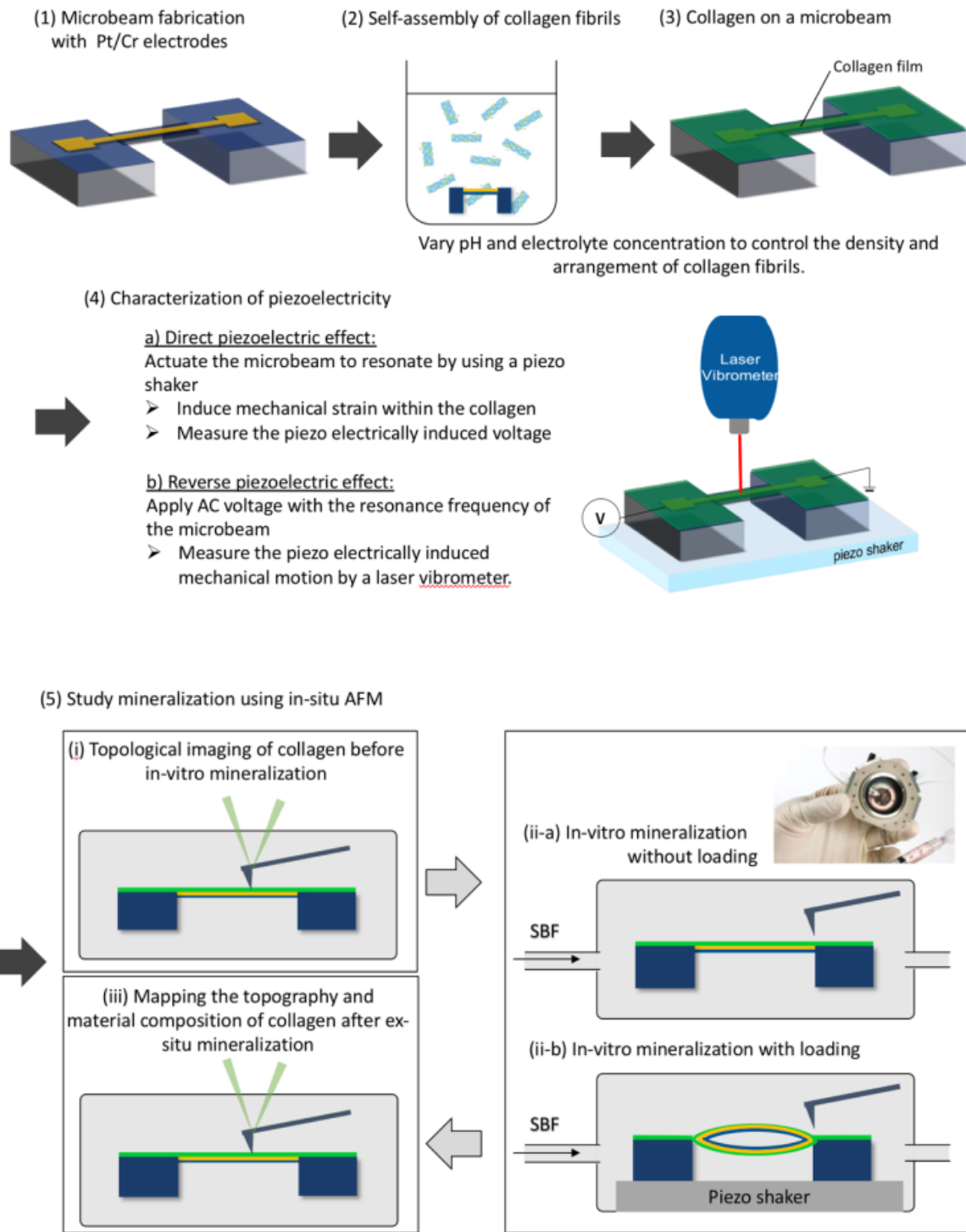


Figure 19: Schematic of Future Experiment.

For step (1) to (3) shown in Figure 19, the collagen fibrils were coated on a micro beam with platinum electrodes following the steps introduced in Chapter 3. Then the piezoelectric effect of collagen fibrils will be characterized by actuating the microbeam system from the base using a piezoelectric shaker to induce the mechanical strain within the collagen film. Then, the electric potential generated by piezoelectricity can be measured by measuring the voltage across the integrated electrode. To characterize the reverse piezoelectricity, a voltage will be applied to the collagen film via the electrode and the motion induced by piezoelectrical mechanical strain will be measured by using a laser Doppler vibrometer. This process is to study how collagen piezoelectricity is affected by the collagen fibrils morphology such as its arrangement, density and orientation. Step (5) shows the process of characterizing collagen mineralization. The collagen self-assembly morphology on the micro beam will first be characterized in a buffer solution before mineralized. Then by injecting the SBF (Simulated Body Fluid, which contains similar mineral contents in our blood) into the environment, the collagen will start to be mineralized while having mechanical motions applied by the piezo shaker (to trigger collagen fibrils piezoelectricity). After the mineralization process is done, the morphology will then be characterized to compare with the structure before mineralization. Therefore, the effect of collagen piezoelectricity on its mineralization process can be then identified.

5.3 Summary

Fibrillogenesis of type I collagen has been characterized by varying environmental conditions (ionic strength, pH, and conductive substrate) with the application of AFM tapping mode imaging. The formation of self-assembled collagen on a highly conductive substrate (platinum) was especially characterized for the need for future applications. The process of

collagen coating on platinum substrates can be applied into future experiment to study the role of collagen piezoelectricity in the mechanism of collagen mineralization.

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